REMARKS

Claims 1-11 currently are rejected. Claim 1 has been amended to narrow the scope of the claim. Claim 11 has been similarly amended and a proviso added. The text of claim 11 also was amended to add clarity to the claim. No new matter was introduced into claim 1 or claim 11 by the amendments and the applicants respectfully request the amendments be made of record.

Claims 1-11 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement.

The Office Action quotes Leyessen *et al.* to support an allegation that at the time the invention was made one skilled in the art would not predict that a compound of formula I would effectively treat a virus of family *Flaviviridae*. This conclusion appears to result from a rather selective reading of the author's conclusions since the very next sentence suggesting the <u>probability</u> of generalized *Flaviviridae* chemotherapy by HCV replication inhibitors was ignored.

> Despite the major clinical impact of flaviviruses such as DENV, JEV and TBEV, there is as yet no drug available for the chemoprophylaxis or chemotherapy of infections with theses viruses. <u>The intense search for inhibitors of HCV replication will probably result in the discovery of compounds that inhibit replication of *Flaviviridae* in general</u>.

P. Leyssen *et al. Clin. Microbiol. Rev.* **2000** 13(1):67-82 (emphasis added)

The Office Action further asserts that at the time the application was filed there were no teachings in the prior art which would lead one of ordinary skill in the art at the time the invention was made to predict that the administration of a compound of formula I would effectively treat a virus of family *Flaviviridae*. However L. Stuyver *et al.* in WO02/48165 published June 20, 2002 disclose compositions and methods for treating *Flaviviridae* (Hepacivirus, Flavivirus and Pestivirus) infections including BVDV and HCV using nucleoside derivatives. Subsequently published applications also support the suggestion that nucleoside inhibitors of HCV NS5B polymerase may be used in other *Flaviviridae* infections. Table 4 of US 2005/00009737 discloses activity of against West Nile Virus, dengue fever and yellow fever.

The Office Action also quotes Leyssen for the proposition that "[t]herapy for pestivirus infections is not believed an option". Applicants note this comment was made with reference to a BVDV infection in swine which is highly contagious and therefore infected herds are slaughtered to

prevent further transmission and because the cost of any treatment would likely exceed the economic value of the animals. This does not imply pestivirus chemotherapy in humans is not an option and, indeed, chemotherapy would appear preferable to the approach taken to control a swine infection.

The Office Action asserts that no *in vitro* data was presented suggesting the viral activity of the instant compounds. Compound I has demonstrated against hepatitis C virus (HCV) activity and is presently in clinical trials for that indication. The other compounds in the instant invention are prodrugs which are transformed to Ia *in vivo*. Claims to the use of Ia and prodrugs thereof to treat patients suffering from HCV have been granted in U.S. Patents 6,784,166 and 6,846,810. The activity against HCV can be found in the table spanning columns 53 and 54 of the '166 patent. Applicants acknowledge the claims in the instant application exclude methods of treating HCV. In order to further exemplify the activity against other *Flaviviridae*, the activity of the triphosphate Ib against dengue fever virus polymerase is provided herewith (TABLE 1).
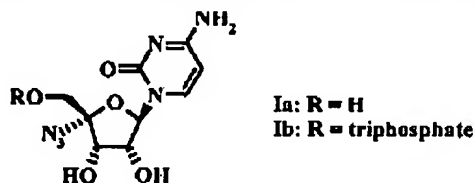

Ia: R = H
Ib: R = triphosphate

TABLE 1		
Compound	IC_{50} (μM, Elongation)	IC_{50} (μM, De novo)
3'-dATP	4.67±2.19	
3'-dCTP	1.49±0.84	2.37
3'-dGTP	0.89±0.71	
3'-dUTP	0.91±0.91	
Ib	1.04±0.54	2.30

Nucleoside inhibitors of viral polymerases generally require *in vivo* phosphorylation of the 5'-hydroxyl to produce the enzymatically active species. The anti-HCV activity is very strong evidence that human nucleoside kinases convert Ia to Ib. Furthermore the conversion of the prodrugs of the present invention to Ia has been established and representative data can be found in '810 patent.

In TABLE 1, 3'-dATP, 3'-dCTP, 3'-dGTP and 3'-dUTP are the 3'-deoxyadenosine, 3'-deoxycytidine, 3'-deoxyguanosine, 3'-deoxyuridine triphosphates respectively which are the positive controls. The activity of Ib was comparable to the positive controls and Ib inhibited both *de novo* transcription and transcript elongation with similar potency. The investigator's report on the activity against dengue virus polymerase is attached (EXHIBIT A).

A person skilled in the art would be a physician specializing in treating infectious diseases. The efficacy of a compound of the present invention in a clinical setting can be determined easily by monitored by overt symptoms or by measuring the production of viral protein or viral RNA. A high dose to quickly inhibit viral replication and minimize the opportunity for the development of resistant strains is commonly used in chemotherapy. When the symptoms subside the dose would then to titrated down to a level which prevented the resurgence of the infection until the virus was completely eliminated.

In the interest of facilitating the prosecution and to overcome the suggestion in the Office Action the claims are overly broad, the claims have been narrowed to prodrugs with optimal pharmacokinetic properties. Reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph, is respectfully requested.

Claim 11 is rejected under 35 U.S.C. §102(e) as anticipated by Devos et al. US 6,784,166.

A proviso has been added to claim 11 requiring that at least one of R^2-R^4 is other than hydrogen. Thus, in the current claims, one of the esters must be an ester or a carbonate. In US 6,784,166 the C5' hydroxyl is explicitly not derivatized and C2' and C3' positions can be hydroxy but not acyloxy (–OC(=O)R) or alkoxy carbonyloxy (-OC(=O)OR). Thus compound 13 in US 6,784,166 no longer anticipates the instant claim.

The Office Action also alleges that compound 27 anticipates the instant claim; however compound 27 has a methoxy substituent at C-3'. The definition of R^3 does not include alkyl, therefore, compound 27 does not anticipate the current claims.

Withdrawal of the rejection under 35 U.S.C. §102(e) is respectfully requested.

CONCLUSIONS

For the reasons herein disclosed the claims as amended are believed to be in condition for allowance and a favorable action and issuance of a notice of allowance is respectfully requested. No fees are believed to be due with this submission but in the event that a fee is required the Examiner is authorized to deduct the fee from our deposit account 18-1700. If the Examiner believes a telephone conference will expedite the prosecution of this application, the Examiner is invited to contact the undersigned at the number indicated below.

Respectfully submitted,

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Enc: Exhibit A

EXHIBIT A

Report on:

Kinetic Properties of Inhibitors of Dengue Viral RNA-Dependent RNA Polymerase

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Introduction

Dengue viruses (DEN, types 1-4) are members of the *Flaviviridae* family of positive-(+) strand RNA viruses and are important re-emerging human pathogens transmitted by mosquitoes, *Aedes aegypti* and *Aedes albopictuus* in tropical and subtropical regions of the world. Dengue viruses, the most common tropical infectious agents after malaria, are causative agents of dengue fever, dengue hemorrhagic fever fever, and dengue shock syndrome affecting about 100 million people globally. DEN2 is the most prevalent of the four serotypes as (6, 7, 11, 16). The dengue fever is a simple self-limiting disease with high fever, severe body ache and muscle pain, and it is usually not fatal and the patient recovers with life long immunity. However, the more severe forms of the dengue viral diseases are the dengue hemorrhagic fever and dengue shock syndrome and of the 500,000 cases, as many as 25,000 deaths reported annually worldwide (6). There is no vaccine or antiviral therapeutics currently available effective against dengue viruses.

The DEN2 genome of New Guinea C strain is 10,723 nucleotides long with a type I 5'-cap structure and no poly(A) tail at the 3'-end (9); for a review, see (3)). The RNA gonome codes for a single polyprotein, NH2-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH, which is processed within the endoplasmic reticulum (ER). The structural proteins that make up the virion (capsid, C; prM, precursor membrane; envelope, E) are produced by cotranslational processing in the ER by signal peptidase. The C-terminal portion of the polyprotein encodes at least seven nonstructural proteins that are produced by different proteases; the NS1-NS2A cleavage by a host ER lumen protease, NS4A-NS4B site by the signal peptidase and the rest are by the viral two-component serine protease, NS2B/NS3 (for reviews, see (3, 20)).

NS5 is the largest of the DEN2 structural proteins and contains conserved motifs found in

several RNA-dependent RNA polymerases (RdRP) of (+) strand RNA viruses in the

carboxy terminal region of NS5 (18, 19). The purified NS5 proteins from DEN1, DEN2,

and Kunjin virus, as well as purified NS5B of hepatitis C virus (HCV) have the RdRP

activity (1, 8, 14, 15, 23, 24). In the amino terminal region of NS5, there are conserved

motifs found in several 5'-RNA methyltransferases (13, 21). The crystal structure of this

methyltransferase domain has been reported (5).

An in vitro viral RNA replicase assay has been developed to determine the

parameters for (-) strand synthesis. This assay utilizes cytoplasmic extracts prepared from

DEN2-infected mosquito (C6/36) or monkey kidney (LLC-MK2) cells and exogenous

viral subgenomic RNA templates containing both 5'- and 3'-terminal regions (25, 26). Of

the two products formed, one was the same size as the template RNA and was RNAse A

resistant and therefore, was double-stranded in nature; the other product was shown to be

a hairpin RNA, twice the size of the template RNA (2x), formed by 3'-end elongation of

a "fold-back" structure of the template RNA. The hairpin RNA also gave rise to an

RNAse-resistant, template-sized product upon digestion of the single-stranded loop

region of the hairpin. The newly synthesized RNA in the RNAse-resistant double-

stranded RNA product was shown to be (-) strand polarity by RNAse H mapping.

In a later study, we expressed the DEN2 NS5 with an N-terminal His tag in

Escherichia coli and purified the protein to near-homogeneity. The purified NS5 also

gave rise to two RNA products, 1x and 2x, in the absence of any other host or viral

proteins. Moreover, the 1x product was shown to be due to de novo synthesis from the 3'-

end of template RNA and the ratio between the de novo synthesis product and the 3'-end

elongation product varied depending on the incubation temperature (1); incubation at lower temperatures promoted de novo (1x) product and the higher temperature favored the 3'-end elongation product (2x). The influence of temperature on the type of products formed could be explained by a model in which the DEN2 polymerase exists in two conformational states, "closed" and "open", that are in equilibrium. When the polymerase is in the "closed" conformation, it is unable to bind to the 3'-end of a "fold-back" structure and is forced to synthesize short primers de novo; these primers are then elongated to form the template size, de novo product by the polymerase through a conformational change to an "open" form. This "open" conformation of the polymerase also preferentially promotes the 3'-end elongation of the template to form the hairpin (2x) product at higher temperatures (1). Thus, by varying the temperature of incubation of the RdRP reaction, it is possible to analyze the parameters influencing the de novo synthesis versus elongation from the 3'-end a primer or "fold-back" structure of the template. A previous study revealed that pre-incubation of the polymerase with the template RNA, ATP and GTP is sufficient for the formation of de novo initiation complex. While the concentrations of ATP, CTP and UTP in the RdRP reaction could be $10\mu M$ for de novo initiation and elongation steps, the concentration of GTP should be high (in the 100-$500\mu M$ range) for de novo synthesis to occur. This high concentration requirement of the dengue viral polymerase for GTP is similar to that of HCV polymerase [Luo, 2000 #1016 ; Zhong, 2000 #1055], Qβ-RdRP (2) and brome mosaic viral polymerase (10). In this study, we use this in vitro RdRP assay system to analyze the effects of chain terminators and nucleoside triphosphate analogs as inhibitors of RNA synthesis.

Materials and Methods

Dengue viral polymerase.

Dengue viral polymerase was expressed in E. coli TOP10 F' cells and purified as

described previously (1). E. coli cells (1 ℓ culture), transformed with pMHA-77-3

plasmid were grown in LB media containing 100 μg/ml ampicillin and 0.5% glucose

(w/v) at 37 °C until A600 nm reached 0.55. Bacteria were then centrifuged at 5,000 x g in

a Beckman HS-4 rotor at 4 °C for 20 min. The pellet was resuspended in LB media

containing 1 mM isopropyl-β-D-thiogalactopyranoside and ampicillin, and incubated for

10 hrs. at 18 °C. Bacteria were pelleted and lysed by French press in 80 ml of lysis buffer

(50 mM NaH_2PO_4, pH 7.0, 300 mM NaCl, 10 % glycerol, 1 % Nonidet P-40, and 1X

Complete™ protease inhibitor mixture without EDTA purchased from Roche Molecular

Biochemicals (Mannheim, Germany). Lysate was then incubated with 1.5 ml of Talon

resin (CLONTECH, Palo Alto, CA) at 4 °C for 1 hr. The resin was batch-washed five

times, with 12 ml of buffer A containing 50 mM NaH_2PO_4, pH 7.0, 300 mM NaCl, 10 %

glycerol. After transfer of Talon resin to a disposable Bio-Rad column, nonspecific

proteins were removed by washing with 40 ml of the buffer A containing 15 mM

imidazole (pH 7.1), followed with 25 ml of 20 mM imidazole in the same buffer. Proteins

were eluted from the Talon resin with buffer A containing 0.5 M imidazole. NS5-

containing fractions were pooled, concentrated by Centricon-30 (Millipore, Bedford,

MA) to ~500 μl, and applied to a G-75 Sephadex (Sigma) column. Proteins were eluted

from the column in 0.5-ml fractions at 5 ml/hr. NS5 was eluted between fractions 21 and

30. Fractions were pooled and dialyzed against 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1

mM dithiothreitol, 5 mM $MgCl_2$, and 40 % glycerol. Purified NS5 protein was aliquoted

and stored at -20 °C.

Preparation of RNA Templates

Contruction of the plasmid encoding DEN2 subgenomic RNA template has been described previously (26). RNA templates were prepared by in vitro transcription catalyzed by T7-RNA polymcrase (Promega) of the PCR product produced by a 5'-end primer containing the T7 promoter-5'-UTR sequences and the 3'-end primer containing the complement of the exact 3'-terminal sequence of DEN2 RNA as described previously (26). RNA was quantified by spectrophotometry and the integrity was verified by denaturing urea-polyacrylamide gel electrophoresis, followed by staining with acridine orange.

RdRP Assay in elongation reaction conditions

The standard reaction mixture (50 μl) contained 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, template RNA (0.3 μg), 10 μM each of ATP, GTP, UTP, and unlabeled CTP, and 10 μCi of $[\alpha\text{-}^{32}P]CTP$ along with 70 ng of purified NS5 except when indicated. The reaction was carried out by incubation at 30 °C for 3 hrs. and terminated by acid phenol/chloroform extraction, followed by ethanol precipitation after the addition of yeast tRNA (5 μg) as a carrier. The RNA pellet was collected by centrifugation, and the pellet was dried. RNA was resuspended in 50 μl of nuclease-free H_2O and passed through a Bio-Rad P-30 column to remove unincorporated nucleotides. Flowthrough fraction was precipitated with ethanol. RNA was analyzed by formaldehyde-agarose gel electrophoresis and visualized by autoradiography (26). The reaction products were analyzed by densititometry utilizing the NIH program ImageJ (http://rsb.info.nih.gov/ij/). RdRP reactions at different temperatures were carried out using a gradient thermocycler

(Tgradient, Biometra, Go¨ttingen, Germany). The reactions were terminated and analyzed as described above.

RdRP Assas in de novo reaction conditions

The standard reaction mixture (50 µl) contained 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM MgCl$_2$, template RNA (0.3 µg), 500 µM each of ATP, GTP, and UTP, 10 µM unlabeled CTP, and 10 µCi of [α-^{32}P]CTP along with 70 ng of purified NS5 except when indicated. The reaction was carried out by incubation at 25 °C for 1 hr. and terminated by acid phenol/chloroform extraction, followed by same procedures described for elongation reaction.

Determination of IC$_{50}$ Values of RdRP Inhibitors

Various concentrations of RdRP inhibitors (0.1 to 100uM, final concentrations) were added to the reaction mixtures, and the amount of product RNA were measured and compared to control sample with no RdRP inhibitor. IC$_{50}$s were calculated from the graph of relative % inhibition vs RdRP inhibitor concentration.

Results

(a) Enzymatic characteristics of RdRP

First, we sought to determine the kinetics of the DEN2 RdRP activity by varying the template RNA concentration as well as whether the polymerase activity was proportional to incubation time. As shown in Fig. 1, the RNA synthesis which was measured as incorporation into 3'-end with varying concentrations of RNA template followed a typical Michaelis-Menten kinetics. The Km and Vmax values calculated from these experiments are: Km, 40.6 nM and Vmax, 1.1 pmole/hour, respectively.
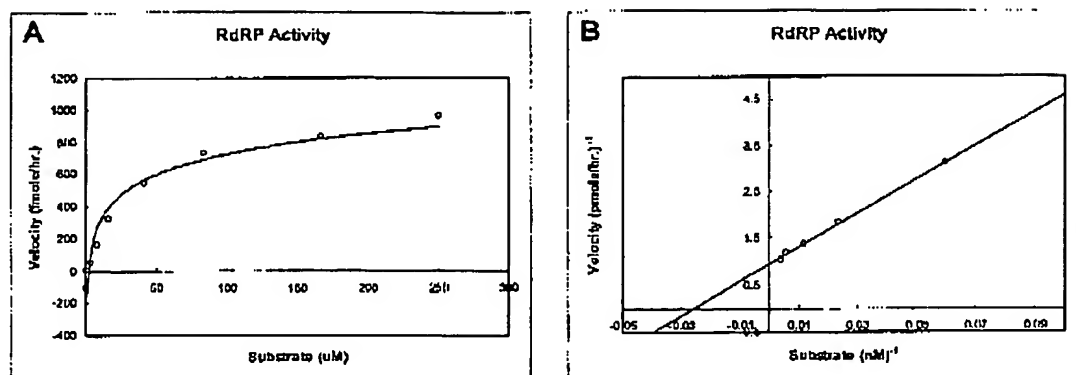
Fig. 1. Enzyme activity of RdRP with varying substrate RNA concentrations

The RdRP activitiy was measured as incorporation of radiolabeled nucleotides at the 3'-end of the template RNA by elongation. A. The values of RNA synthesis were plotted versus the concentrations of substrate RNA used. B. Lineweaver-Burk plot from which the Km and Vmax of the polymerase for the subgenomic RNA template were calculated.

Next, we examined the optimum incubation time for the polymerase assay in the absence

and the presence of R█████████-TP inhibitor. As shown in Fig. 2, the activity increased

proportionally with reaction time up to 120 min either in the absence of the inhibitor

(indicated as "No inhibitor) or in the presence of R█████████-TP inhibitor.

Fig. 2. RdRP activity in elongation in the absence and presence of R████████-TP inhibitor.
The standard RdRP activity assay was carried out in the absence (o-o) and presence (●-●) of inhibitor at various time points and the total radioactivity corresponding to RNA synthesis was plotted against time.
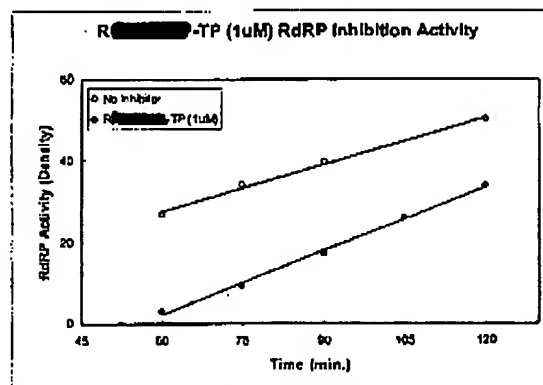
In order to find out the concentration range of the polymerase required for synthesis of

RNA within a linear range of activity under a specific incubation time, the RdRP assays

were performed by adding various amounts of the polymerase in the assays which were

then incubated at two different incubation times, 105 and 180 min.
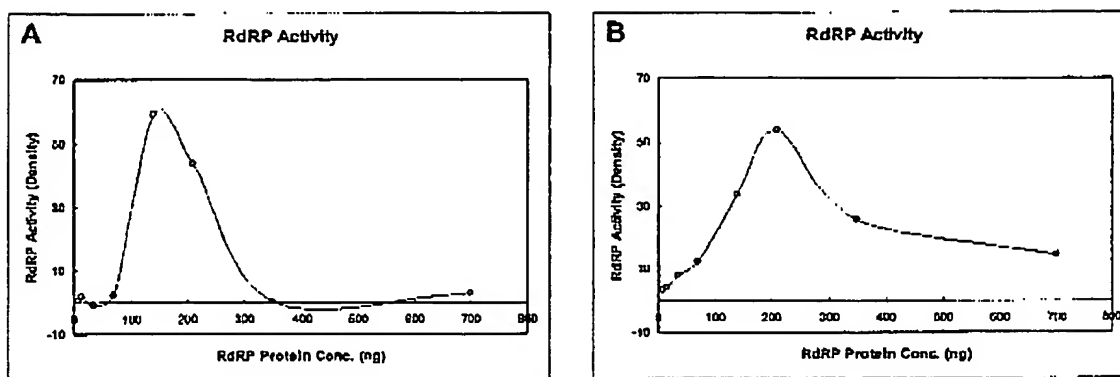
Fig. 3. Determination of optimum enzyme concentration and incubation time

The RdRP assays (elongation conditions) were carried out under standard assay conditions as described under Materials and Methods except by adding various amounts of the polymerase. The reaction mixtures were incubated either for 105 min (A panel) or 180 min (B panel).

As shown in Fig. 3 (A and B), the polymerase activity peaked at a protein concentration of about 140ng in 105 min. reaction time (Fig. 3A), whereas 200ng was required in a 3 hr. incubation (Fig. 3B). The lag in the first four (low) enzyme concentrations is possibly due to the time required for formation of active replication complex which seems to be overcome at longer incubation periods (Fig. 3B) or at higher enzyme concentrations (Fig. 3A, 140 ng). Subsequent experiments were done using 140ng of the enzyme and 105 min of incubation period.

(b) Determination of IC_{50} values of the polymerase with inhibitors in elongation reaction

Two groups of RdRP inhibitors were tested to determine the inhibitor concentrations that bring about 50% inhibition of RdRP elongation reaction; in the first group were the 3'dNTPs and in the second group, the Roche compound, R██████-TP, was the only one tested (Fig. 4, A-E).

From the results shown in Fig. 4, the JC_{50} of R██████-TP was calculated to be

1.04µM. Among the 3'-dNTPs, 3'-dGTP was the most effective and 3'-dATP was the

least (Table 1). The IC_{50}s of 3'-GTP and 3'-dATP were 0.89 µM and 4.67 µM,

respectively.
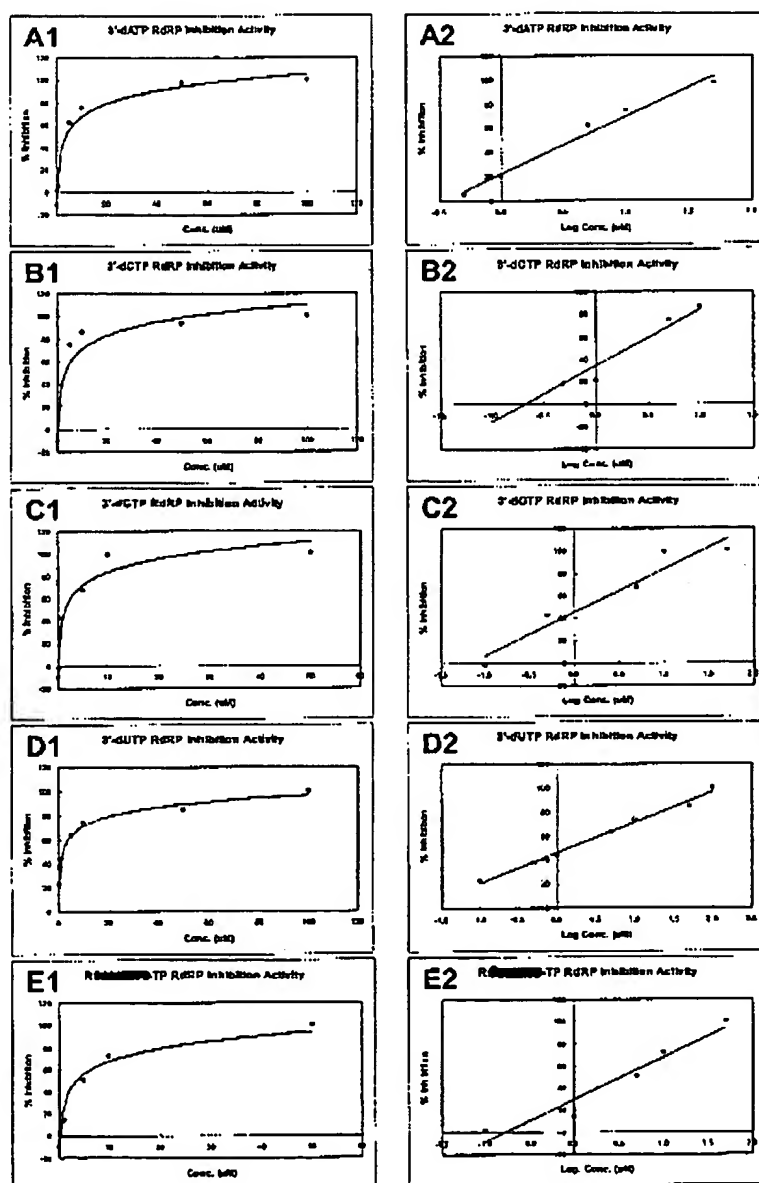
Fig. 4. The IC$_{50}$ values of various RdRP inhibitors. Elongation of RNA synthesis at the 3'-end of template RNA was measured for each of the inhibitor at various concentrations and the % inhibition values were plotted. A1, B1, C1, D1, and E1 are the inhibition activities of 3'-dATP, 3'-dCTP, 3'-dGTP, 3'-dUTP, and R████████-TP, respectively. A2, B2, C2, D2, and E2 are the corresponding semi-log plots of the A1, B1, C1, D1, and E1, respectively.

Table 1.. IC$_{50}$s of RdRP inhibitors in elongation and de novo synthesis conditions.

The IC$_{50}$ values for inhibition of elongation reaction were calculated from the Fig. 4, A-E. [1,2] The values for elongation reactions represent an average of three experiments whereas those for *de novo* initiation was only from a single experiment. The concentrations of 3'-dCTP and the R████████-TP required for 50% inhibition of de novo synthesis were also determined by carrying out the RdRP assays at 25°C (1).

Inhibitor	IC$_{50}$ (Elongation)[1] (µM)	IC$_{50}$ (De novo)[2] (µM)
3'-dATP	4.67 ± 2.19	
3'-dCTP	1.49 ± 0.84	2.37
3'-dGTP	0.89 ± 0.71	
3'-dUTP	0.91 ± 0.22	
R████████-TP	1.04 ± 0.54	2.30

(c) Determination of IC$_{50}$ values of the DEN2 polymerase with 3'-dCTP and R████████-TP in *de novo* synthesis of RNA.

The in vitro RdRP assay for DEN2 polymerase permits analysis of kinetic properties of the enzyme in elongation reaction as well as in de novo synthesis of RNA by varying the temperature of incubation from 35oC in the former to 25°C in the latter (1). We determined the IC$_{50}$ values of the polymerase for the 3'-dCTP and the R████████-TP at

25°C. The IC_{50} values were calculated from the results shown in Fig. 5 to be 2.37μM and

2.30 μM, and respectively (Table 1, Fig. 5). These IC_{50} values were about two-fold

higher from those for the elongation reactions.
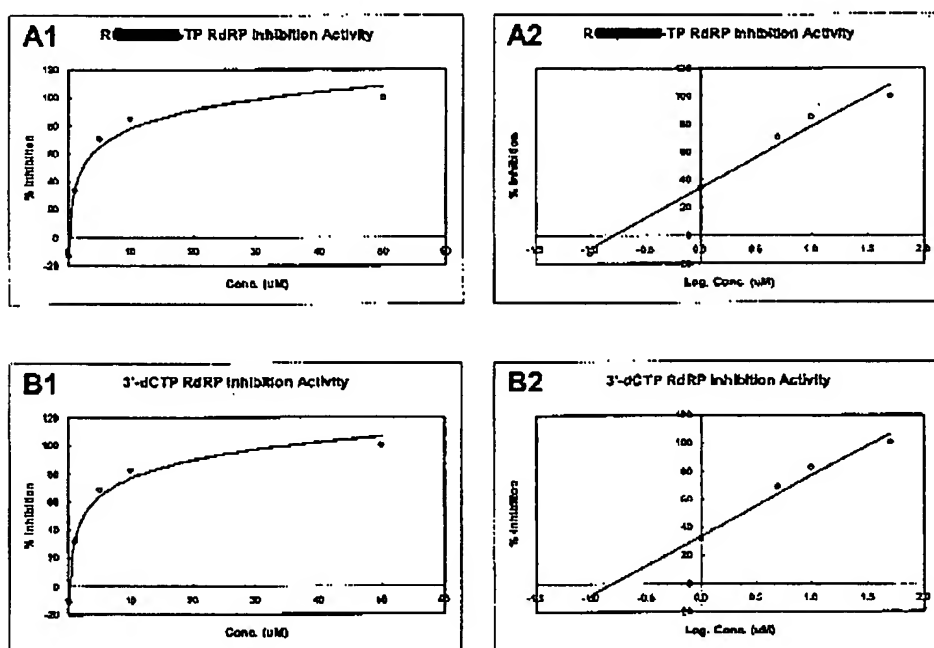
Fig. 5. Inhibitory activities of R██████-TP and 3'-dCTP in de novo synthesis of RNA by the DEN2 polymerase.
The polymerase activities in de novo synthesis were determined and quantified from the radiolabeled, template-sized (1x) product at each concentration of the inhibitor. A1 and B1 represent the inhibition activity of R██████-TP and 3'-dCTP. A2 and B2 are the corresponding semi-log plots of the data in A1 and B1.

DISCUSSION

The in vitro RdRP assays using the DEN2 polymerase were performed using the

subgenomic RNA template which contains all the conserved regulatory elements present

in the viral genome within the 5'- and 3'-noncoding sequences as well as the 5'-

cyclization motifs within the N-terminal coding sequence of capsid protein which has

been shown to be important for DEN2 minus strand RNA synthesis in vitro ((1, 26), and

Kunjin and Yellow fever viral RNA replication in cultured mammalian cells in vivo (4, 12). This assay system has also been used to study the role of 3'-stem loop structure which is highly conserved in all flaviviral RNAs and a conserved pseudoknot structure present in DEN2 and West Nile viral RNAs (25). Since there are close correlations between the conclusions reached from study of the mutational effects of the 3'-stem loop structure observed in vivo (27) and the replication efficiencies of the templates observed in vitro (25), this assay system exhibits a high degree of template specificity and allows distinction between inhibition of 3'-end elongation and de novo synthesis steps in viral RNA replication. This characteristic can be exploited to identify the mode of action of an inhibitor Moreover, by using the minus strand template in the vitro system, we are able to study the requirements for (+) strand RNA synthesis and could potentially also study the effect of inhibitors on (+) strand RNA synthesis.

In this study, we used the 3'-dNTPs and R███████-TP, an analog of CTP as substrate-based inhibitors of the DEN2 polymerase. The 3'-dNTPs have been used as chain terminators of DNA polymerases as well as DNA-dependent RNA polymerases such as T7, SP6, and Ø6 polymerases. Some of the purine or pyrimidine nucleoside chain terminators with modification on the ribose ring which are in clinical use are acyclovir for herpes infection, zidovudine (AZT) and didanosine (ddI) for AIDS, and lamivudine and adefovir for HBV infection (Shim et al. 2003). These analogs lack 3'-OH for elongation of nucleic acid synthesis and thus function as chain terminators by incorporation into a growing chain of DNA or RNA. However, only limited studies have been carried out using the 3'-dNTPs as inhibition of RdRPs encoded by a number of human pathogens such as flaviviruses, hepatitis C virus, influenza virus, poliovirus,

rhinovirus and respiratory syncytial virus. In this study, the four 3'-dNTPs were examined as chain terminators of RNA synthesis by the DEN2 polymerase. The apparent K_m values of the DEN2 polymerase for ATP and GTP were found to be 2.25 ± 0.84 and 0.37 ± 0.07 µM, respectively (17). The HCV polymerase exhibits Km values for ATP in the range of 2.34 ± 0.07 µM (15) to 10 ± 2 µM (22) and for GTP, in the range of 1.85 ± 0.28 µM to 0.23 ± 0.05 µM. The Km of the DEN2 polymerase for GTP and ATP are also close to that of HCV polymerase (0.35 ± 0.07 and 2.25 ± 0.84 µM, respectively) (17). Among the four 3'-dNTPs, 3'-dCTP, 3'-dGTP, and 3'-dUTP all have similar IC_{50} values for inhibition of elongation, but the IC_{50} value for 3'-dATP is about 3-4 fold higher (Table 1). The R█████-TP, a CTP analog also has a similar IC_{50} value as that of these three 3'-dNTPs. In order to examine whether the 3'-dNTPs are inhibiting the RNA synthesis by incorporation followed by chain termination, we need to perform single nucleotide incorporation assay using a short template-primer system. This is feasible because we have recently shown that the DEN2 polymerase could utilize a short synthetic primer, AGAA, very efficiently on the subgenomic RNA template.

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